

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/86, C07K 14/18, C12N 15/62, A61K 39/245, 39/29, 31/70, 9/127 // C12N 15/87, A61K 48/00, 39/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/27069</b> <b>(43) International Publication Date:</b> 12 October 1995 (12.10.95)
<b>(21) International Application Number:</b> PCT/EP95/01080 <b>(22) International Filing Date:</b> 22 March 1995 (22.03.95)  <b>(30) Priority Data:</b> 9406498.7 31 March 1994 (31.03.94) GB  <b>(71) Applicants (for all designated States except US):</b> SMITHKLINE BEECHAM BIOLOGICALS (S.A.) [BE/BE]; 89, rue de l'Institut, B-1330 Rixensart (BE). BIOPTION AB [SE/SE]; Ringvägen 38, S-141 31 Huddinge (SE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DALEMANS, Wilfried [BE/BE]; SmithKline Beecham Biologicals (S.A.), 89, rue de l'Institut, B-1330 Rixensart (BE). BRUCK, Claudine [LU/BE]; SmithKline Beecham Biologicals (S.A.), 89, rue de l'Institut, B-1330 Rixensart (BE). LILJESTRÖM, Peter, Torsten [FI/SE]; Bioption AB, Ringvägen 38, S-141 31 Huddinge (SE).  <b>(74) Agent:</b> DALTON, Marcus, Jonathan, William; SmithKline Beecham, Corporate Intellectual Property, SB House, Great West Road, Brentford, Middlesex TW8 9BD (GB).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ALPHAVIRUS RNA AS CARRIER FOR VACCINES  <b>(57) Abstract</b>  Vaccine compositions are provided that employ alphavirus RNA molecules containing exogenous RNA sequences encoding an antigen for direct administration to a patient.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## ALPHAVIRUS RNA AS CARRIER FOR VACCINES.

The present invention is related to polynucleotides, in particular to recombinant polynucleotides which form part of a vector, especially an alphavirus vector. The invention also relates to the recombinant vectors and to pharmaceutical compositions comprising the vectors which are suitable for vaccine use.

WO 90/11092 ('Expression of exogenous polynucleotide sequences in a vertebrate') describes a pharmaceutical product comprising naked polynucleotide, operatively coding for a biologically active peptide in a suitable form for injection into a tissue to cause the cells of the tissue to express the said polypeptide. In particular it is claimed that the peptide may be immunogenic and the 'naked DNA' which codes for it may be used to vaccinate, for example, humans.

It has been reported that it is possible to immunise mice with 'naked DNA' to protect them from influenza virus (see *Science*, Volume 259, 19th March 1993, page 1691).

In WO 92/10578 Garoff and Liljestrom described an expression system based on alphaviruses, in particular Semliki Forest Virus (SFV). SFV has a single stranded RNA genome of positive polarity and replication of this capped and polyadenylated RNA starts upon the initial translation of the 5' two thirds of the genomic RNA, producing a polyprotein which by autoproteolytic events is post-translationally cleaved into four non-structural proteins (nsP1 - nsP4). These proteins are responsible for the replication of the plus strand genome into full length minus strands which later in infection are copied into new plus strand genomes. For further details, see also *Biotechnology*, Volume 9, December 1991, pages 1356 - 1361.

WO 92/10578 describes a DNA molecule encoding protein sequences being inserted into engineered variants of the cDNA of a positive stranded RNA virus genome from alphavirus which then, via RNA transcription and transfection into tissue culture cells, is used to produce recombinant virus particles for either immunisation or protein production. In relation to immunisation, the recombinant RNA genome (structural alphavirus protein genes replaced by heterologous gene) was cotransfected into target cells in vitro with another RNA directing expression of alphavirus structural proteins. This led to encapsidation of the recombinant RNA. Resulting particles were used for vaccination.

The present approach, uses naked alphavirus RNA (with insertion of heterologous protein gene in place of structural alphavirus genes) for direct immunisation by administration to a mammal. This naked RNA may optionally admix with lipid for stabilisation purposes, but is not necessarily encapsulated by the lipid. Alternatively the RNA molecule may be delivered on an inert, in particular a

gold, particle by means of a gene gun (see US Patent No. 5100792, 5036006 and 4945050). The inventors have demonstrated that it is possible to obtain good expression of the antigen in the muscle using RNA either stabilised by lipid, or without lipid being present. The term naked is used herein to distinguish the vaccine composition of the present invention from RNA molecules encapsidated by viral proteins, since in the present invention, RNA molecules are directly administered to the target tissue in vivo.

According to the present invention there is provided a vaccine composition comprising a naked RNA molecule derived from an alphavirus RNA genome and capable of efficient intracellular replication in an animal host cells, which RNA molecule comprises the complete alphavirus RNA genome regions, which are essential to replication of the said alpha virus RNA, and further comprises an exogenous RNA sequence capable of expressing its function in said host cell, said exogenous RNA sequence being inserted into the region of the RNA molecule which is non-essential to replication thereof, together with a suitable carrier, diluent or pharmaceutically acceptable excipient.

There is also provided a method of preventing or treating a viral infection in a mammal (especially a human) by administering an effective dose of the vaccine composition according to the invention.

There is further provided the use of a naked RNA molecule derived from an alphavirus RNA genome and capable of efficient intracellular replication in an animal host cells, which RNA molecule comprises the complete alphavirus RNA genome regions, which are essential to replication of the said alpha virus RNA, and further comprises an exogenous RNA sequence capable of expressing its function in said host cell, said exogenous RNA sequence being inserted into the region of the RNA molecule which is non-essential to replication thereof, in therapy, more specifically for the preparation of vaccine composition for use in the prevention or treatment of a range of infections in a mammal. The vaccine composition according to the invention may find application in anticancer vaccine therapy as well, wherein the exogenous sequence will encode a tumor antigen.

This is the first medical use of such naked RNA molecules and accordingly the invention in one aspect provides an RNA molecule as herein described for use in medicine.

In a preferred embodiment the alphavirus is Semliki Forest Virus (SFV).

Preferably the naked RNA molecule which may be used in the invention has an exogenous RNA sequence which encodes a protein, a polypeptide or a peptide sequence defining an exogenous antigenic epitope or determinant.

The naked RNA molecule which forms part of the composition according to the invention may be prepared according to the methods set forth in WO 92/10578.

In a particular embodiment the exogenous RNA sequence is derived from influenza haemagglutinin in (HA) DNA, for example as illustrated in the Example below. In an alternative embodiment, the exogenous sequence is derived from a HSV DNA, in particular a sequence which codes for an HSVgD protein or derivative thereof.

To prepare such an embodiment there is required a vector (circular or linearised) comprising alphavirus DNA and an exogenous DNA fragment derived from the exogenous antigen of interest such as influenza HA or HSVgD. For example DNA corresponding to the coding sequence of influenza HA protein from strain A/PR/8/34 (Winter, G et al, 1981 Nature, 292, 72-75) can be ligated to a linearised SFV plasmid such as pSFV1 (Liljestrom, P and Garoff, H (1991), Biotechnology, 9, 1356-1361, prepared as described hereinbelow. Such vectors form a further aspect of the invention.

The compositions of the invention may be administered using the dosages and routes of administration described in WO 90/11092. It will be apparent however that the precise dosage will depend on factors such as the weight sex, mode of administration, general health of the patient and the condition to be treated. Nonetheless for Intramuscular use the dosages to employed will typically be in the range of 0.05µg/kg to about 50 mg/kg, more typically from about 0.1 to 10 mg/kg. Subcutaneous, epidermal, intradermal or mucosal administration are also possible.

The following examples illustrate the invention.

#### **EXAMPLE 1: GENERATION OF ANTI-INFLUENZA HA ANTIBODIES AND A PROTECTIVE RESPONSE UPON INTRAMUSCULAR INJECTION OF NAKED RECOMBINANT SFV-HA RNA IN MICE.**

##### **DNA construction.**

Recombinant DNA technology was applied according to standard procedures. Those are well-known by persons skilled-in-the art, and these general procedures are referred in Sambrook et al. (1).

The DNA corresponding to the coding sequence of the influenza haemagglutinin (HA) protein from strain A/PR/8/34 (2) was excised from pMS2 by restriction digest with *HindIII*, and protruding ends were blunt-ended by filling-in with Klenow polymerase. Remaining pUC plasmid DNA was further digested with *PvuI*, *NdeI*, and *BglI*. These insert fragments were subsequently cloned into *SmaI* linearised pSFV1 (3), and transformed into *E. coli* XL1-Blue competent cells. Recombinant colonies bearing the HA insert were detected by colony screening. First,

colonies were hybridised to oligonucleotide WD19 (GGGGCAATCAGTTTCTGG) specific for the HA coding sequence. Subsequently, the colonies were re-hybridised to the oligonucleotide WD14 (GGCGGTCCTAGATTGGTG) specific for pSFV vector sequence. DNA of colonies hybridising to both oligonucleotides was prepared, and  
5 further analysed by *EcoRI* restriction digest. Clones with the HA insert in the right orientation were further analysed by restriction digests with *PvuII*, *RsaI*, *BamHI+SphI*, *BglII+PstI* and *BamHI+EagI*. A large-scale preparation of recombinant clone pSFV-HA14 was prepared and analysed by restriction digest with *EcoRI* and *XhoI*. The junctions created by ligation of the HA insert into pSFV1 were  
10 analysed by DNA sequencing. Primers that were used were WD14 (see above), WD18 (GCCTATACATATTGTGTC; corresponding to HA sequences), and WD19 (see above). The first 70 N-terminal amino acids of the HA protein were identical to the published sequence (2) except for a Cys to Ser mutation of residue 10 in the signal peptide sequence. Two additional, silent mutations were detected at the DNA level for  
15 residues Val36 and Leu67. Sequencing of the 3' junction evidenced an additional CC dinucleotide preceeding the *HindIII-SmaI* junction, which is however outside the coding region.

#### RNA preparation.

20 pSFV-HA14 DNA was linearised by restriction digest with *SpeI*, and purified. Linear plasmid was incubated with SP6 RNA polymerase in the presence of the four ribonucleotides, Cap analogue, recombinant RNase-inhibitor, and the necessary buffer components according to (3). For *in vivo* application, the *in vitro* transcribed RNA was subsequently purified by Sephadex G50 chromatography, and concentration of  
25 the RNA was determined by UV absorbance. The integrity of the transcript was verified by agarose gel-electrophoresis.

#### *In vitro* expression of HA protein.

Four µg of recombinant pSFV-HA14 RNA was transfected into BHK cells  
30 ( $4 \cdot 10^6$  cells) by electroporation. Total cell lysate was harvested 16 hours after transfection, and proteins equivalent to  $5 \cdot 10^4$  cells were separated by poly-acrylamide gel-electrophoresis. HA protein was evidenced by Western blot analysis with the monoclonal antibody H308 which is specific for the influenza HA protein.

#### 35 Mouse injection with naked SFV-HA RNA.

30µg of pSFV-HA14 RNA (per mice) was first incubated for 10 minutes at room temperature with 5µg of lipofectin reagent (GIBCO-BRL) and then adjusted to a final volume of 50µl of physiological saline solution (PBS). A sample of this

preparation was analysed by agarose gel-electrophoresis, and the integrity of the RNA in the final preparation was confirmed. Twenty CB6F1 mice (four groups of five mice) were injected into the quadriceps muscle, each with 50µl of RNA preparation (30µg RNA). 21 days post-injection, a first serum sample was taken (retroorbital; post-I) and mice were reinjected with 50µl RNA preparation now containing 22.9µg of RNA. 21 days after this booster injection, a second serum sample was taken (post-II).

#### ELISA for anti-HA antibodies.

Serum samples from post-I and II bleedings were tested by an ELISA. Microtiter plates were coated overnight at room temperature with a 1/8000 dilution (in PBS) of sucrose-purified Influenza virus A/PR/8 (lot 07/092/412). After rinsing four times with PBS + 0.1% Tween20, plates were saturated with a solution of 1% gelatine in PBS (1 to 1.5 hour at 37°C). After rinsing (see above), the respective mouse serum samples were applied. These samples were diluted in PBS + 0.1% Tween20 + 0.1% gelatine (dilution starting at 1/25 or 1/100 for post-I and II samples, respectively, and serially diluting by a factor 3), and were incubated for 1.5 hours at 37°C. After four rinses, a 1/3000 dilution of a biotinylated anti-mouse antiserum (Amersham RPN1001) was applied and incubated for 1 hour at 37°C. After four rinses, plates were incubated with a 1/3500 dilution of Extravidine (Sigma E2886) (40 minutes, 37°C). After three rinses (see above), plates were once rinsed with water, and OPD substrate (Sigma 8787) was applied. Reaction was done at room temperature for 20 minutes (in the dark), and stopped by addition of 50% (vol) 2M H2SO4. Read-out of the colour reaction was done at the wavelength of 492nm. Mouse S.31.3 had a titer of 74 EU/ml at post-I (figure 1), whereas the other 19 mice injected tested negative (figure 1 for mice S.31.1-2-4-5, other results not shown). The anti-HA ELISA titer of mouse S.31.3 increased to 176 EU/ml upon boosting (post-II sample; figure 2). Positive control, influenza infected mice had an average titer of 7000-8000EU/ml in the same test (42 days post-infection), and naive mice tested negative (figure 3).

#### PROTECTION EXPERIMENTS

##### Experiment 1

Three weeks after the second injection, the mice were challenged with 10<sup>7.28</sup> TCID<sub>50</sub> of the homologous A/Pr/8 influenza strain given intranasally. Mice from both vaccination groups were sacrificed at days 1,3,5 and 7 post challenge (5 mice per group and per day). Their lungs, trachea and turbinates were collected, triturated and stored at -80°C until assayed. Protection of the mice was assessed by quantifying the

amount of virus present in the lungs at each time points mentioned above using a classical cell culture titration technique.

Results of the lung titration are given in the annexed table 1 ( $\log_{10}$  TCID<sub>50</sub>/organ). They indicate that vaccination of mice with RNA encoding HA is able to provide extensive protection against influenza replication in the lungs of vaccinated/challenged animals.

These results demonstrate for the first time that injection of naked recombinant RNA into animals can elicit protection against viral replication in an animal model. Since only one out of the twenty animals tested developed a detectable antibody response as monitored by ELISA, it seems that the observed protection is not mediated by pre-existing antibodies, but rather by priming of the immune system and possibly also by a cellular immune mechanism.

#### Experiment 2

Mice (Balb/c, 15 mice per group) were injected into the tibialis cranialis muscle with 10 $\mu$ g SFV RNA (control RNA prepared from pSFV1 without insert) or 10 $\mu$ g SFV-HA RNA (see above) contained in 50 $\mu$ l PBS. A booster injection was given three weeks later, and animals were challenged as described above. Non-immunised, challenged animals served as controls. ELISA analysis (see above) of serum samples taken 3 weeks after booster injection showed that all animals which received SFV-HA RNA had seroconverted. Virus titers in the lungs and trachea at days 3, 4, and 5 post-challenge (5 animals per point) were determined as described, and are represented in figure 4. These results show that a very consistent reduction in viral titer can be obtained upon immunisation with naked SFV-HA RNA. Protection against viral infection of the lungs was complete.

#### References.

1. Sambrook, J. et al.. (1989) "Molecular Cloning. A laboratory manual. Second edition." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Winter, G. et al.. (1981) Nature, 292, 72-75.
3. Liljeström, P. and Garoff, H.. (1991) BioTechnology, 9, 1356-1361.

#### Example 2: Expression of beta-Galactosidase upon intramuscular injection of naked SFV3-lacZ RNA.

SFV3-lacZ RNA was prepared by *in vitro* transcription of SpeI linearised pSFV3-lacZ DNA (1). For intramuscular injection, 12 $\mu$ g of this RNA was resuspended in 1xPBS buffer in a final volume of 50 $\mu$ l (per injection point). Two groups of each five mice



were injected with 50µl RNA in the tibialis muscle and 50µl RNA in the quadriceps muscle of the opposite leg. Injection of this RNA did not lead to any visible adverse effect in the mice. In order to detect expression of the beta-Galactosidase protein, mice were sacrificed at day 1 and day 3 post-injection (5 mice per sampling) and the injected muscles were dissected. A lysate was prepared from both tibialis and quadriceps muscles, and beta-Galactosidase activity was assayed enzymatically (2). Expression of the reporter gene was detected in all the injected tibialis muscles, both at day 1 and 3 post-injection. Mean enzymatic activity was 13.7 and 27.3U/mg protein, respectively. Expression in the quadriceps was detectable in 3 out of 5 mice at day 1 post-injection (mean activity 1.6U/mg protein), and in 1 of 5 mice at day 3.

#### References:

1. Liljeström P and Garoff H (1991). *Bio/Technology* 9, 1356-1361.
2. MacGregor GR et al (1991). In: *Gene transfer and expression protocols. Methods in molecular biology* 7. Murray EJ (ed). Humana Press, Clifton, New Jersey. pp 217-235.

#### Example 3 : Generation of antibodies against HSV2-gD by intramuscular injection of naked recombinant SFV-gD RNA.

Recombinant SFV-gD RNA was produced in a comparable way as described in example 1. Briefly, the fragment encoding the HSV2 gD protein (in a truncated form covering the extracellular part of the protein from amino acid 1 to 307) (1) was isolated from pUC12-gDStop clone 26 by EcoRI digestion. After filling-in the protruding ends, the fragment was ligated into SmaI digested pSFV1, and recombinants were selected after transformation into *E. coli*. The recombinant plasmid, pSFV-gD, was linearised by partial digest with SpeI, and SFV-gD RNA was obtained after in vitro transcription. Biological activity of the RNA was confirmed by transfection into BHK21 cells revealing the expression of a truncated gD protein by Western blot.

Anaesthetised mice (Balb/c) were injected into the tibialis muscle with different amounts of SFV-gD RNA in PBS in a final volume of 50µl. Two booster injections were given after 1 and 5 months, respectively. Serum samples were taken at different time points after the RNA injections and antibodies directed against HSV-gD were quantified by a standard ELISA (see also example 1). Briefly, microtiter plates were coated with purified gD protein (1µg/ml), and subsequently saturated with PBS containing 4% newborn calf serum and 1% bovine serum albumin. Serum samples

were applied at different dilutions, and thereafter incubated with a biotinylated anti-mouse IgG (Amersham RPN1177). Detection was performed by adding Streptavidin-POD (Amersham RPN1051) followed by OPDA (Sigma P8787). Specific anti-gD antibody titers were expressed as  $\mu\text{g/ml}$  total IgG by reference to a mouse total IgG standard curve. Figure 5 shows the results of SFV-gD naked RNA immunisation. All animals seroconverted, and a clear booster response can be observed.

#### Reference.

- 10 (1) McGeoch D.J. et al. (1987). J. Gen. Virol. 68, 19-38.

TABLE 1

Protective effect of naked RNA vaccination against replication of influenza virus  
in the lungs of challenged mice

	Day 1	Day 3	Day 5	Day 7
pSFV 1HA	<1.9 <1.9 <1.9 <1.9	<1.9 <1.9 1.9 <1.9 <1.9	<1.9 <1.9 <1.9 <1.9 <1.9	<1.9 <1.9 <1.9 <1.9 <1.9
pSFV 1	<1.9 <1.9 <1.9 4.55 <1.9	<1.9 <1.9 4.05 <1.9 <1.9	2.3 <1.9 2.8 1.9 <1.9	<1.9 <1.9 <1.9 <1.9 <1.9

(Units are log<sub>10</sub> TCID<sub>50</sub>. Each data point represents an individual mouse. pSFV1 : control mice injected with RNA without HA coding sequences.)

**Claims**

1. A vaccine composition comprising an alphavirus RNA molecule containing an exogenous RNA sequence encoding an antigen.
2. A vaccine composition as claimed in claim 1, wherein the RNA molecule is formulated with lipid.
3. A vaccine composition as claimed in claim 1 wherein the RNA molecule is absorbed on to an inert particle.
4. A vaccine composition as claimed in any of claims 1-3 wherein the exogenous RNA sequence encodes an Herpes Simplex antigen.
5. A vaccine composition as claimed in any of claims 1-3 wherein the exogenous RNA sequence encodes an Influenza antigen.
6. A vaccine composition as claimed in any of claims 1-3 wherein the exogenous RNA sequence encodes a tumor antigen.
7. An alphavirus RNA molecule comprising an exogenous RNA sequence encoding for an Herpes Simplex antigen or influenza antigen.
8. An alphavirus RNA molecule as claimed in claim 7 wherein the antigen is an HSVgD antigen or an influenza haemagglutinin antigen.
9. A DNA molecule corresponding to the RNA molecule of claim 7 or 8.

Figure 1/5

**ELISA of post-I serum samples.**

(Serum samples of five mice injected with 30µg SFV-HA14 RNA were taken 21 days after injection. Mouse S.31.3 has an ELISA titer of 74EU/ml. The values on the X-axis correspond to ELISA dilutions, and EU titer is determined as the intersection of the Y value with the cut-off line. The cut-off value was determined on ELISA titers of mice recovered from influenza A/PR/8 infection (positive controls; see figure 3). )

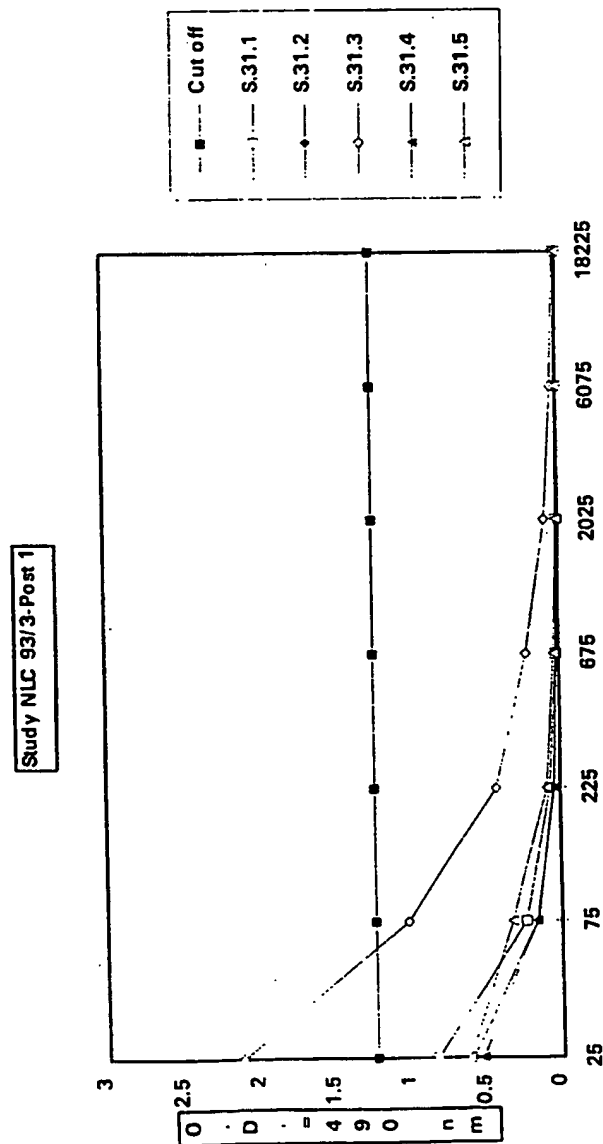
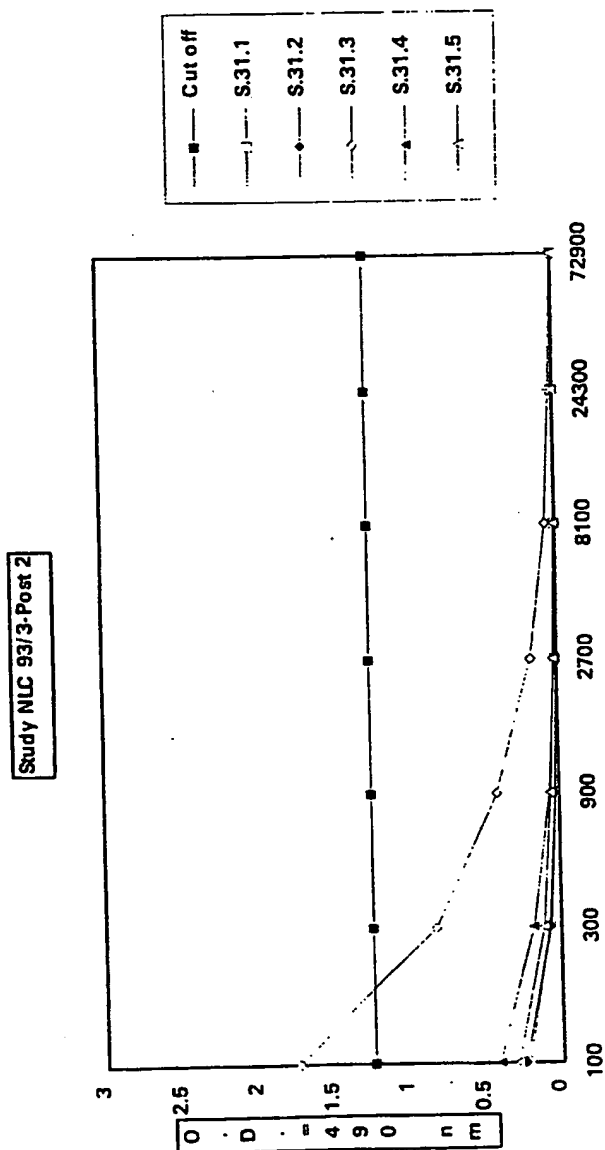


Figure 2/5

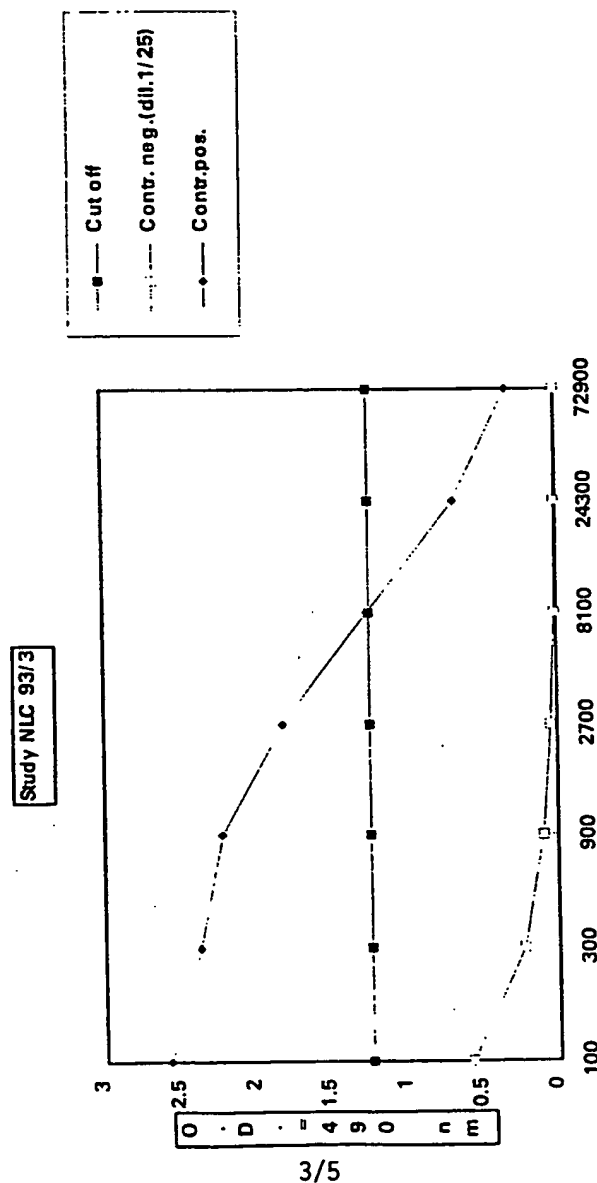
## ELISA of post-II serum samples.

(Serum samples of five mice injected twice with SFV-HA14 RNA were taken 21 days after the second injection. Titers are determined as in figure 1, and mouse S.31.3 has now a titer of 176EU/ml.)



**Figure 3/5****ELISA of control infected and naive mice.**

(Positive controls (Contr.pos.) were mice infected with influenza virus A/PR/8, 42 days post-infection. The 50% of maximal optical density was taken as the cut-off value, which is also used as the standard cut-off value for other ELISA read-outs (figures 1 and 2). Negative controls (contr.neg. (dil.1/25)) were naive mice; values presented correspond to 4 times more concentrated serum samples, i.e. serum dilution started at 1/25 rather than the 1/10 as indicated for the positive controls.)



**Figure 4/5**

Figure 4. Virus titers upon challenge with Influenza virus in SFV-HA immunised animals versus controls. (SFV RNA: control RNA derived from pSFV1 vector; SFV-HA RNA: recombinant RNA derived from pSFV-HA14, encoding the haemagglutinin protein of Influenza virus A/PR/8/34; control: non-immunised mice; Cum titer: cumulative titer of recovered viruses from the trachea and the lungs of the three experimental groups (15 animals per group)).

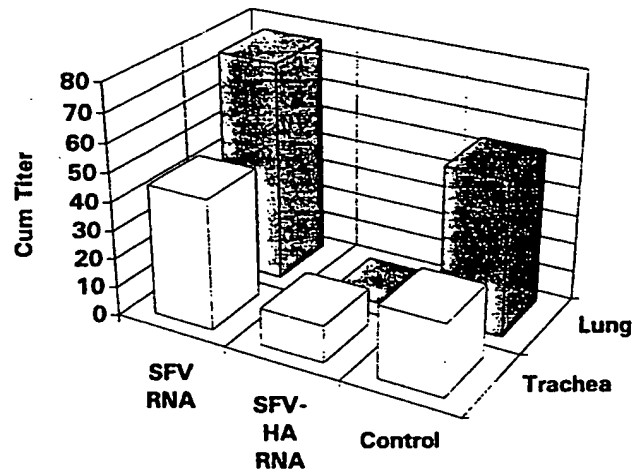
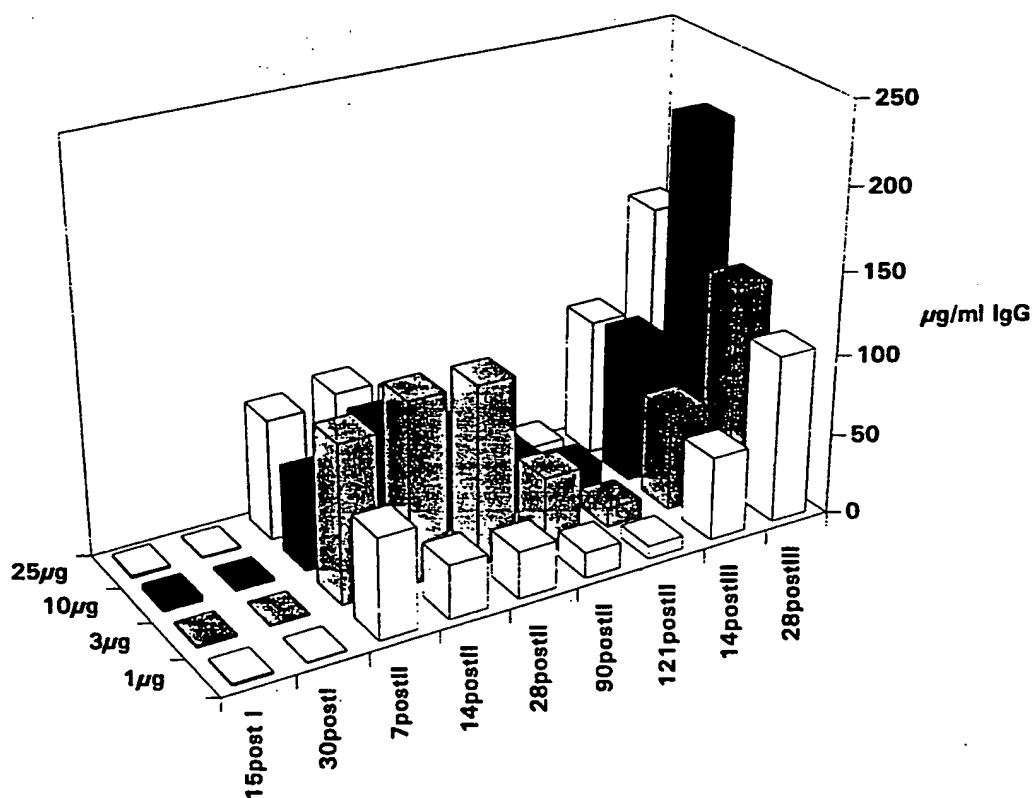




Figure 5/5

Figure 5. Specific anti-HSV-gD antibodies upon immunisation with SFV-gD RNA. (1 - 25 $\mu$ g : amounts of naked RNA injected; 15postI - 28 postIII : serum samples taken 15 or 28 days, respectively, after primo (postI) or second booster (postIII) injection, respectively;  $\mu$ g/ml : values of specific anti-HSV-gD total IgG.)



# INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/EP 95/01080

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/86 C07K14/18 C12N15/62 A61K39/245 A61K39/29 A61K31/70 A61K9/127 //C12N15/87, A61K48/00, A61K39/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIO/TECHNOLOGY (1991), 9(12), 1356-61, LILJESTROEM, P. ET AL. 'A new generation of animal cell expression vectors based on the Semliki Forest virus replicon'	1,9
Y	see page 1359, right column, paragraph 1 see page 1361, left column, paragraph 2 ---	1-9
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, April 1992 WASHINGTON US, pages 2679-2683, HAHN, C. ET AL. 'INFECTIOUS SINDBIS VIRUS TRANSIENT EXPRESSION VECTORS FOR STUDYING ANTIGEN PROCESSING AND PRESENTATION'	7-9
Y	cited in the application see the whole document ---	1,5,7-9
-/-		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search  16 June 1995		Date of mailing of the international search report  29.06.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer  Andres, S

## INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/EP 95/01080

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO-A-90 11092 (VICAL INC ;WISCONSIN ALUMNI RES FOUND (US)) 4 October 1990 cited in the application see page 6, line 28 - page 7, line 23 see page 13, line 10 - page 15, line 28 see page 35, line 30 - page 41 see examples 1,5-7,11 ----	2,4,6
Y	US-A-4 945 050 (SANFORD JOHN C ET AL) 31 July 1990 cited in the application see column 6, line 35 - column 7, line 13 see claims ----	3
X	AIDS RES. HUM. RETROVIRUSES 9(10), 963-70, October 1993 PAUL, N. ET AL. 'Expression of HIV-1 envelope glycoproteins by Semliki Forest virus vectors' see the whole document ----	1,9
X	US-A-5 091 309 (SCHLESINGER SONDR A ET AL) 25 February 1992 see the whole document ----	1
P,X	VACCINE 12 (16). 1510-1514, December 1994 ZHOU, X. ET AL. 'Self-replicating Semliki forest virus RNA as recombinant vaccine.' see the whole document ----	1,5,7-9
T	AUSUBEL, F. et al. (eds.): 'CURRENT PROTOCOLS IN MOLECULAR BIOLOGY'; volume 2; 1995, J. WILEY & SONS, INC. UNIT 16.20 see page 16.20.1 - page 16.20.6 -----	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat Application No

PCT/EP 95/01080

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9011092	04-10-90	AU-A- 5344190 EP-A- 0465529 JP-T- 4504125	22-10-90 15-01-92 23-07-92
US-A-4945050	31-07-90	US-A- 5100792 US-A- 5036006 US-A- 5371015	31-03-92 30-07-91 06-12-94
US-A-5091309	25-02-92	NONE	